Eukaryon

Volume 6 *Evolving Connections* Article 29

3-1-2010

Spinal Cord Regeneration: Ready, Set, Nogo

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Spinal Cord Regeneration: *Ready, Set, Nogo*

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Abstract

Neurons in the peripheral nervous system (PNS) have the capability to regenerate after injury or disease while central nervous system (CNS) neurons do not. Elucidation of the reasons for this difference in regenerative ability is crucial to developing treatments for sufferers of CNS disorders, injury, and stroke. Therefore, my lab investigates molecular mechanisms underlying neuronal repair, axonal guidance, and growth cone formation and collapse. We discovered the Nogo protein and its inhibitory role in CNS repair. The Nogo protein interacts with the Nogo-66 receptor (NgR), located on the axons of CNS neurons, to inhibit axonal sprouting after injury. Blockage of this interaction by a competitive inhibitor allows limited CNS axon regeneration in vitro and in spinal cord injury model mice. During normal development and repair, axonal pathfinding is mediated by the growth cone, which is guided by extra cellular cues that repel or attract the extending axon. This extension is the result of continuous polymerization and depolymerization of the actin skeleton. My lab works to elucidate the mechanism behind how these growth cones are guided, and we have uncovered a number of important steps in this pathway. Understanding the inhibitory environment to CNS regeneration is essential to developing treatments for its disorders.

Introduction

Central nervous system (CNS) injury, such as spinal cord injury (SCI), stroke, and neurodegenerative disease contribute heavily to the number of people suffering from disabilities in the United States. In 2008, there were over 250,000 Americans living with disabilities resulting from spinal cord damage, and that number will grow by 12,000 cases each year[1]. Over 5 million people suffered from stroke in 2005, and it is estimated that one stroke occurs in the United States every 40 seconds[2]. Impairment due to SCI typically stems from mechanical forces that damage spinal cord neurons while stroke leads to ischemia in brain tissue, resulting in cell death. Neurodegenerative disease results in the progressive loss of neurons in the CNS, ultimately leading to symptoms characteristic of the disease[3]. For example, Parkinson's Disease (PD) results in loss of neurons in the substantia nigra, an area essential for voluntary movement[3]. Thus, PD patients exhibit movement based symptoms that stem from CNS neuron loss. In all cases, the neurons lost are irreplaceable. Regeneration of lost CNS neurons is impaired by the endogenous inhibitors. Consequently, sufferers of CNS injury usually do not recover substantially from their disability. Treatment typically consists of physical therapy, which does result in minor improvement, but significant impairment still exists[3]. A to CNS repair found in the brain and spinal cord. number of inhibitory factors contribute to the lack of regeneration in the CNS. After injury to CNS neurons, astrocytes and glial cells are attracted to the site. Astrocytes promote the formation of a glial scar, which inhibits regeneration of the neuron. In addition, the myelin of the supporting oligodendrocytes contains proteins that interact with receptors on CNS neurons to inhibit growth[3].

Interestingly, the peripheral nervous system (PNS) faces no such obstacles to recovery following injury. After damage to the PNS, macrophages rapidly and efficiently clear the resulting myelin debris, and genetic changes triggered in the cell lead to axonal extension through a structure called the growth cone. Guided by extracellular cues released by supporting Schwann cells, the regenerating axon is guided into its correct position based on attraction or repulsion to the cues[3].

My lab focuses on regenerative and developmental aspects of the nervous system. We seek to explain how axons are guided into their proper position during development and repair through better understanding of growth cone biology. A second aim is to elucidate the reasons for the differential regeneration ability between CNS and PNS neurons. A better understanding of these processes is essential for improving current treatment of CNS disorders.

Our contributions to the field exist on two fronts. The exact component of CNS myelin that prevented regeneration was unidentified until 2000[4,5,6]. Along with two other labs, we first discovered the myelin associated protein Nogo, which has been found to contribute heavily to inhibition of CNS repair. Further investigation into Nogo has revealed much about its inhibitory role in regeneration, including the receptor it interacts with and the structural features that mediate Nogo and receptor contact[7]. Blocking this interaction has lead to improved function in SCI model mice[8,9,10].

Our second contribution results from the work conducted on the molecular mechanisms behind axonal guidance and growth cone sprouting. Both intracellular and extracellular regulators of neuronal extension exist. My lab characterized the functional mechanism of GAP-43, an intracellular regulator of axonal growth[11]. We have also provided insight into the semaphorin family of extracellular growth regulators[12].

The Path To Nogo

The difference in regeneration of CNS and PNS neurons has long puzzled the scientific community. It was not until the late 1980s that the possible source of inhibition was discovered. Martin Schwab's lab discovered that oligodendrocytes and CNS myelin inhibit growth of CNS neurons[13]. Further investigation proved the source of this inhibition stems from the presence of a 35-kd and 250-kD protein fraction (NI-35 & NI-250) that could be isolated from the myelin. When damaged CNS neurons were exposed to myelin lacking these fractions, no inhibition was observed[14]. Schwab's lab later went on to develop the IN-1 antibody, which binds to the NI-250 protein and its inhibitory effects[15]. Furthermore, treatment of SCI mice models with the IN-1 antibody led to axonal regeneration[16]. However, the exact identity of this protein and the gene that encodes it were still unknown.

In 2000, our lab was one of three groups that identified the gene coding for NI-250. The transcription

*This author wrote the paper for Biology 480: Neural Frontiers (Senior Seminar) taught by Dr. Shubhik DebBurman.

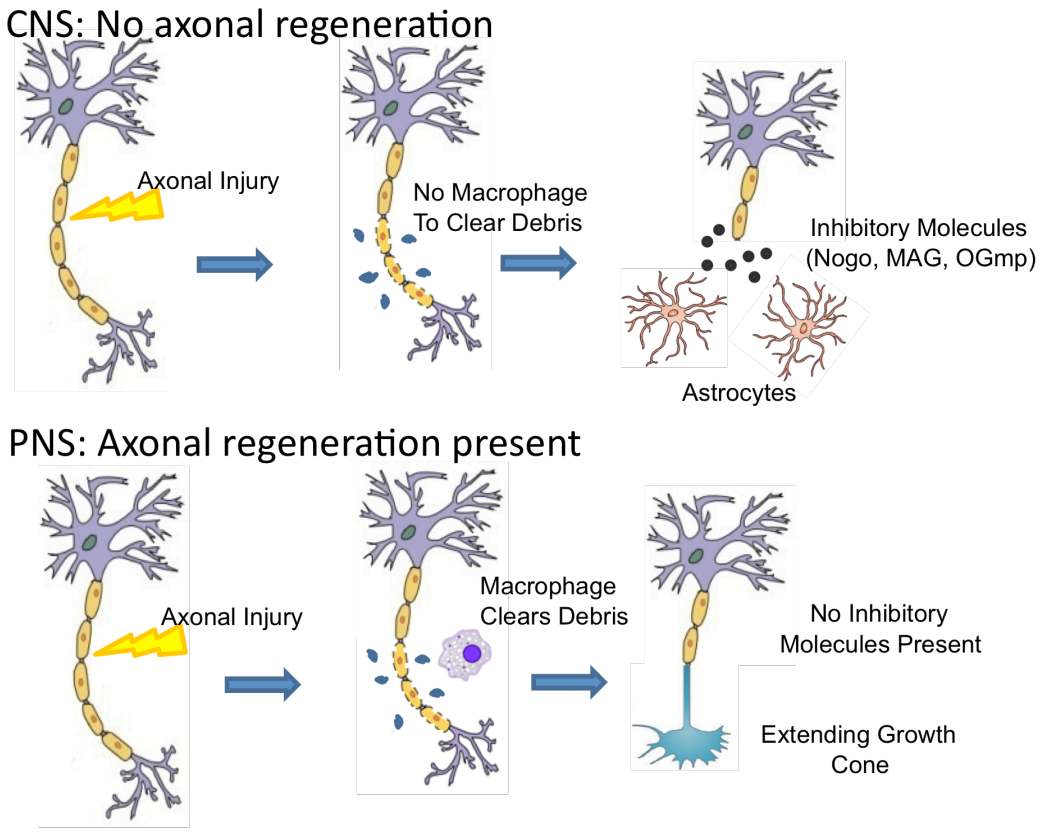

Figure 1: Regeneration in the CNS vs. PNS Axonal regeneration is limited in the CNS as compared to the PNS. Damage to the axon results in the deposition of myelin debris. In the PNS, this debris is cleared rapidly by macrophages. However, no macrophages are present in the CNS to clear the debris, impeding neuronal repair. In addition, CNS damage attracts astrocytes, which form a glial scar that prevents regeneration. No astrocytes are present in the PNS to induce scarring. CNS neuron regeneration is also hindered by inhibitory components in the myelin such as Nogo, MAG, and OMgp, all of which inhibit neuronal sprouting. The PNS has no such inhibitory molecules, which allows for the extension of the growth cone and axonal sprouting.

product of the suspect gene believed to code for NI-250 was exposed to IN-1 antibody. The IN-1 protein readily bound to NI-250. Additionally, we were able to isolate the cDNA coding for this protein and name it Nogo. The newly described protein is a member of the Reticulon family, and the gene codes for three forms of Nogo: Nogo-A, Nogo-B, and Nogo-C. Nogo-A strongly inhibited chick dorsal root ganglion (DRG) outgrowth and was found to be expressed mainly in oligodendrocytes and CNS myelin[4,5,6]. Thus, Nogo is a major contributor to the non-regenerative environment of the CNS. However, the receptor that Nogo interacts with remained unidentified.

Nogo Interacts with the Nogo-66 Receptor

Nogo-A is a transmembrane protein that displays a 66 amino acid (aa) loop to the extracellular environment while the carboxyl region projects into the cytoplasmic space of the cell[4,5,6]. The amino terminal domain of the protein also projects toward the extracellular space. During the initial discovery of Nogo, the 66aa loop domain (Nogo-66) was found to be a potent inhibitor of axonal extension[4]. This extracellular segment's structure and function alluded to the idea that the Nogo protein interacts with a receptor of some kind. Using an alkaline-phosphatase linked Nogo-66 protein, we established that Nogo strongly interacts with a protein found primarily in the brain. This protein is characterized by a high number of leucine repeats, and its presence was required for sprouting inhibition[8]. Hence, this protein interacting with Nogo-66 was named the Nogo-66 Receptor (NgR).

Determining the NgR protein's location was the first step in elucidating the molecular basis for Nogo-66 and NgR interaction. If the NgR protein was found to be expressed on the outside of neurons, it would provide evidence that the Nogo protein, which is found on oligodendrocyte myelin, directly interacts with it. Immunostaining of spinal cord samples from chicks demonstrated that NgR is expressed mainly on the axons of these cells, which supports a model of Nogo-NgR interaction mediated by oligodendrocyte-neuron contact[8]. Thus, it appears after injury that contact between the oligodendrocyte and injured neuron results in an interaction between NgR and Nogo to prevent axonal regeneration.

The next step was to establish that the Nogo-66 and NgR interaction indeed causes growth cone collapse. The NgR receptor was found to be crucial in preventing axonal sprouting through its interaction with Nogo. Retinal ganglion cells from developing chick embryos were transfected with the gene coding for the NgR receptor. These neurons, now expressing the NgR receptor, were exposed to Nogo-66 protein. Outgrowth of the retinal ganglion cells was severely limited upon exposure to Nogo-66 due to growth cone collapse[18]. This protein is GPI linked to the membrane of neurons, and cleavage of this GPI

linkage allows for axonal sprouting even in the presence of Nogo-66[18]. Axonal damage leads to an increase in Nogo levels in oligodendrocyte myelin while NgR levels remain unaltered[17]. The increase around the lesion site contributes to the inhibition of neuronal extension in this area[18]. Upon axonal damage, Nogo interacts with the 66aa extracellular domain of Nogo the NgR receptor to inhibit axonal extension.

NgR is Not Selective to Nogo-66

In addition to Nogo, the NgR receptor interacts with a number of other myelin associated proteins. In 1994, McKerracher et al found that myelin associated glycoprotein (MAG) inhibited neuronal outgrowth after injury[19]. In 2002, Wang et al discovered a second molecule, OMgp, which inhibited axonal extension in a manner similar to MAG[20]. In search for a commonality between these three inhibitory molecules, we assessed to what extent MAG and OMgp interact with the NgR receptor.

Using an AP binding assay, we found that MAG and OMgp bind to the NgR receptor and not the NgR2 or NgR3 receptors[21]. In addition, the binding of MAG and OMgp requires the same leucine rich repeat region that is necessary for Nogo-66 binding. This finding was somewhat surprising, as MAG and OMgp share no sequence homology to each other or to Nogo. MAG and OMgp deficient mice both exhibit slightly increased axonal regeneration after SCI, indicating that these two myelin associated proteins are also major contributors to the lack of regeneration seen in CNS damaged neurons[19,20].

The interaction of NgR with MAG, OMgp and Nogo is also dependent on a coreceptor, discovered by Wang et al in 2002. As stated before, NgR is a GPI anchored protein. Thus, it does not extend functional domains beneath the cell membrane and must function through a coreceptor to inhibit axonal outgrowth after binding its substrate. Previous research demonstrated that p75 is required for inhibition of axonal repair by MAG, but that MAG does not interact directly with the receptor. Using a p75 knockout mice model, we found that signaling by NgR requires the presence of the p75 receptor[22]. The discovery of this interaction provides yet another target to treat CNS impairment.

Interruption of the Nogo/NgR Interaction Promotes Recovery after SCI in Mice

We next began investigating how interrupting the Nogo/NgR receptor interaction could enhance spinal cord injury using a Nogo-66 antagonist peptide, NEP1-40. The NEP1-40 antagonist peptide does not trigger growth cone collapse of chick DRG neurons. This peptide competitively binds to the NgR receptor and blocks the effect of Nogo. Therefore, we used NEP1-40 to block Nogo in an *in vitro* and *in vivo* model system. *In vitro,* chick DRG neurons grown on inhibitory CNS myelin treated with the antagonist peptide did not experience inhibited axonal sprouting[8]. *In vivo,* SCI model mice that received intrathecal NEP1-40 exhibit increased CST sprouting through the site of the lesion, and perform statistically better in motor function tests as compared to untreated SCI mice[8].

The next step was to create lines of mice lacking Nogo to evaluate their ability to regenerate after SCI. At the time, two other labs were pursuing the same goal, albeit each took a slightly different approach. Our lab and Marc Tessier-Lavigne's lab utilized a Nogo A/B deficient strain of young adult mice[9,23]. Our colleague Martin Schwab utilized a Nogo knockout in which all three isoforms (Nogo-A, Nogo-B, and Nogo-C) were absent[10].

Contradictory results were obtained from the three labs. Mine and Martin Schwab's lab found that absence of either Nogo A or Nogo A/B increased sprouting of CST neurons across the lesion in SCI model mice[9,10]. However, some additional differences were apparent. The regeneration of CST fibers near the lesion shown by my lab was much more substantial than the regeneration shown by Dr. Schwab's experiments. Interestingly, the Nogo A -/- mice used by Dr. Schwab also exhibited elevated levels of Nogo-B. It is possible that this increase in Nogo-B attenuates the lack of Nogo-A, and thus can explain the slightly decreased regenerative capacity as compared to the Nogo A/B -/- mice utilized by our lab. To our surprise, Marc Tessier-Lavigne's lab found no such regeneration using a similar mouse model as us[23]. Their Nogo A/B -/- mice showed no evidence of increased regeneration. Two explanations can possibly clear the confusion generated by these experiments. Firstly, the genetic backgrounds of the mice utilized by all three labs were not identical. Slight differences in gene expression could easily explain the differences in regeneration. Secondly, the age of the mice used by the researchers was not controlled for across all three experiments. Our lab demonstrated that older mice exhibited less pronounced regeneration after SCI as compared to younger mice[9,10,23]. Thus, both age and genetic background can explain the differences seen by ours and Dr. Tessier-Lavigne's lab.

The NgR protein also proved to be instrumental in controlling regeneration after SCI. We developed a function blocking NgR ectodomain protein, dubbed NgR(310)ecto-Fc. The spinal cord of rats was then damaged, followed by subsequent treatment of the NgR(310)ecto-Fc protein. After treatment, CST fibers began extending across the lesion site and even began forming synaptic connections to their target neurons[24]. The treated mice also regained substantial motor function, as demonstrated by significantly higher BBB scores compared to untreated mice. Electrical activity of the neurons of treated mice more closely reflected normal neurons as well[25]. Thus, the Nogo/NgR receptor interaction appears to be crucial in controlling recovery of CNS neurons after injury.

Two Fronts of Nogo Inhibition

We previously established that Nogo-66 has an inhibitory role on the outgrowth of extending neurons due to its interaction with NgR. Although the Nogo protein clearly interacts with the NgR receptor and p75 coreceptor, the downstream pathway that ultimately results in growth cone collapse is not understood. The Rho family consists of a number of small GTPase proteins. Previous research demonstrated that activation of Rho or Rho's downstream effector, ROCK, results in diminished neuronal sprouting[26]. Growth cone collapse requires coordinated rearrangement of the actin cytoskeleton. Rho GTPases have been found to be intimately involved in such actin transformations. This knowledge led us to investigate the relationship between Rho and Nogo-66 inhibition.

PC12 cells expressing the NgR receptor were treated with Nogo-66, and levels of GTP bound Rho were assessed. Elevated levels of GTP bound Rho were found in response to Nogo-66 treatment. In addition, blockade of the Rho pathway using the pyridine derivative Y-27632 resulted in axonal extension even in the presence of Nogo-66[26]. Thus, it appears that Nogo-66 activates the NgR receptor, which associates with the p75 receptor. This NgR/p75 complex then activates the Rho pathway, ultimately leading to a signaling cascade which results in collapse of the growth cone due to actin depolymerization.

Interestingly, PC12 exposure to amino Nogo did not activate the Rho pathway. Blockage of the Rho pathway using the Y-27632 followed by subsequent DRG neuron

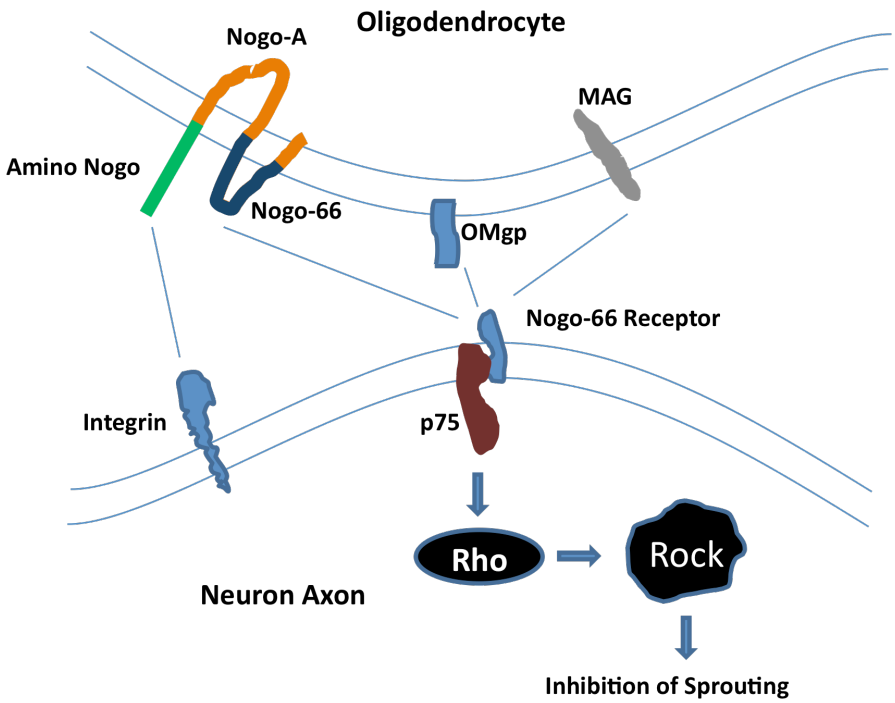

Figure 2. Oligodendrocyte Myelin Components Inhibit Axonal Regeneration: Nogo-66, OMgp and MAG are myelin associated proteins that interact with the NgR receptor to inhibit axonal sprouting of neurons. Binding of these myelin components to the NgR receptor recruits p75 and results in the subsequent activation of the Rho pathway. Ultimately, axonal sprouting is inhibited. Amino Nogo disrupts αv and α5 integrin to disrupt axonal sprouting and cell adhesion.

exposure to amino Nogo still resulted in growth cone collapse[26]. In addition, we found that amino Nogo does not interact with the NgR receptor to inhibit axonal growth[7]. These findings lead us to believe that the inhibitory effects of Nogo can be attributed to a second mechanism mediated by result, we decided to evaluate the interaction between amino-Nogo and the integrin family of proteins. COS-7 cells exposed to amino Nogo had decreased levels of phosphorylated focal adhesion kinase (FAK), indicating integrin activation was suppressed. In addition, chick DRG neurons plated on fibronectin, an activator of integrin, and treated with amino Nogo failed to sprout axons[27]. Moreover, we found amino Nogo to directly inhibit αv and α5 integrin subunits[27]. Thus, Nogo inhibition of axonal sprouting occurs by two mechanisms: Nogo-66 interacts with the NgR receptor to activate the Rho pathway, and amino Nogo disrupts integrins to inhibit cell adhesion and axonal extension (figure 2).

Nogo-66 Receptor is Involved in Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disorder characterized by the accumulation and aggregation of extracellular amyloid-beta (Aβ) protein[28]. These Aβ plaques are thought to contribute to death of the neurons. Interestingly, abnormal axonal extension, which we have shown is mediated by the NgR receptor, is found to occur near Aβ accumulations. Therefore, we investigated the role of the NgR receptor in Aβ deposition. We assessed the accumulation of Aβ protein in APPswe/PSEN-1(ΔE9) Alzheimer's model mice lacking the NgR receptor (-/-). Deletion of the NgR receptor resulted in increased Aβ plaque formation[28]. In addition, treatment of APPswe/PSEN-1(ΔE9) mice with NgR(310)ecto-Fc protein lessened the formation of Aβ plaques. Alzheimer's model mice treated subcutaneously with NgR(310)ecto-Fc also decreased Aβ the amino terminus of the protein. Previous research demonstrates that amino Nogo inhibits cell adhesion of neurons and non-neuronal cells[27]. It also inhibits neuronal outgrowth, and amino Nogo antibodies can attenuate this effect. Integrins play a vital role in cell adhesion, and as a levels in cortical cells and improved spatial memory of the mice. Their performance on a radial arm maze was significantly better than their untreated counterparts[28]. Thus, NgR is clearly an intriguing target for treatment of Alzheimer's disease, and further research is needed to fully elucidate its potential as a drug candidate.

Potential Treatment of CNS Injury

Current treatments for CNS injury are severely lacking. However, our research has uncovered a number of potential treatment avenues. The Nogo and NgR pathway provide several targets alone. Inhibition of Nogo-66 binding using the NEP1-40 antagonist peptide or blocking the NgR receptor using NgR(310)ecto-Fc protein both show promise in treating SCI in mice[8,24]. The blockage of the NgR receptor is most likely to produce the best results, as MAG and OMgp also bind here to inhibit extension[21]. Thus, simply blocking Nogo binding will not remove all obstacles to regeneration. In addition, the amino terminus of Nogo inhibits axonal regeneration by an integrin specific mechanism and would also have to be blocked to allow for unrestricted regeneration[27]. A final method to enhance regeneration would be disruption of the Rho pathway. Since binding of Nogo-66 leads to the activation of the Rho pathway and actin depolymerization, interruption of this activation could allow neuronal extension[26]. One potential problem with this approach is that the Rho pathway is involved in more than just actin depolymerization, and thus side effects of this blockage could manifest in other abnormalities.

Intracellular and Extracellular Regulators of Growth Cone Structure

In the PNS, no myeline associated proteins exist that inhibit growth cone formation after axonal trauma as in the CNS. Indeed, evidence has accumulated over the past 15 years that supports the presence of both intracellular and extracellular regulators of growth cone formation and guidance. One such intracellular factor is the protein GAP-43[29]. This protein is found to be highly concentrated in the membrane of extending growth cones, but the structural basis for this localization during development and injury was unknown. My lab identified the membrane targeting signal located on the N-terminal region of the protein[30]. The function of GAP-43 also remained to be revealed. We determined that the GAP-43 protein increases GTP binding to Go, a G-signaling protein that is found localized with GAP-43 on extending axons[31]. GAP-43 regulation of Go was a hypothesized mechanism of how GAP-43 controls neuronal plasticity. G protein signaling is one known mechanism for changes in axonal growth[31]. To test this, we injected GAP-43 into oocytes of *Xenopus laevis* to determine its effect on G signaling. We found that, upon injection, oocytes became up to 100 times more sensitive to G protein agonists[11]. Thus, it appears that GAP-43 modulates neuronal extension by increasing the sensitivity of G proteins to cellular signaling. In crude terms, it acts as an amplifier of these signals, sensitizing the cell to the cues for growth cone structural change.

The regulation of GAP-43 is controlled by two post-translation modifications: palmitoylation and phosphorylation[32]. Using GAP-43 mutants lacking regions critical for the protein's amplification effect, we determined that palmitoylation of GAP-43 does not allow for amplification of signal transduction. Unexpectedly, we determined that phosphorylation of serine-41is necessary for GAP-43 activity[32]. Thus, GAP-43 amplification of signal transduction occurs though sensitization of G-protein signaling and is controlled by both palmitoylation and phosphorylation of GAP-43.

Extracellular regulators of growth cone formation also exist. One family of extracellular regulators is the semaphorin family. This family includes the collapsin protein, discovered in 1993[33]. While collapsin is known to induce growth cone collapse, the mechanism behind this collapse was not well understood. We first described the protein mediating collapse, CRMP-62, after discovering its cDNA in 1995[33]. We injected collapsin treated *X. laevis* oocytes with chick DRG membrane extracts. Certain fractions of this membrane extract triggered a response by the oocytes to collapsin, and the cDNA of the protein in this fraction was cloned. The isolated protein shared a homologous section with UNC-33, a protein required for axonal guidance in nematode CNS development[33]. Thus, the collapsin interacts with CRMP-62 protein to induce growth cone collapse.

Actin depolymerization is characteristic of growth cone collapse. The rho pathway has previously been implicated in restructuring of the actin network in neurons[12,34]. Thus, we evaluated the interaction of the collapsin protein with rac1, a member of the rho small GTPase family. We discovered that collapsin interacts with rac1 to cause growth cone collapse[34]. In search of a receptor, we found that the collapsin protein binds to a plexin/neuropillin receptor[35]. Similar to Nogo, collapsin collapses growth cones via two mechanisms. The first is through interaction with the Rho signaling pathway to trigger destabilization of actin[12]. The second is an increase in endocytosis of f-actin at the membrane of the growth cone. Typically, the amount of endocytosis and exocytosis at the growth cone membrane is relatively equal. Upon collapsin treatment, endocytosis of growth cone membrane is enhanced, leading to further collapse of the extending axon[36,37]. Thus, the collapsin protein causes collapse of growth cones through rearrangement of the actin cytoskeleton brought about by binding to the plexin/neuropillin receptor and subsequent Rho pathway activation. It also enhances endocytosis of f-actin at the membrane of growth cones to inhibit axonal extension as well.

Conclusion

Regeneration of neurons in the CNS is limited in comparison to the PNS. The molecular basis for these differences remained a mystery since the 1980s. Our contribution to the field comes from the elucidation of the components involved in generating the restrictive regenerative atmosphere of the CNS. The identification of Nogo and the NgR receptor revealed a major contributor to suppression of neuronal outgrowth. In addition, we have determined that the NgR is involved in more than just Nogo binding, and also interacts with the inhibitory proteins MAG and OMgp to prevent axonal regrowth[6,21]. In addition, we have shown that manipulation of the Nogo/NgR system has potential therapeutic applications in mice[16,17]. In addition to CNS injury brought on by trauma, we have begun to investigate the role of the NgR receptor in Alzheimer's[28]. Thus, a wide spectrum of CNS disorders including trauma, stroke, and neurodegenerative disease are intimately linked to the Nogo and the NgR. Treatment of these disorders requires further research into the pathways involved to develop safe, effective treatments for CNS maladies.

Axonal guidance is crucial for proper structure of the nervous system. Both intracellular and extracellular regulators of axonal guidance exist. The intracellular regulator GAP-43 was of unknown function in the early 1990s. My lab determined that the GAP-43 protein amplified G protein signal transduction, ultimately resulting in the restructuring of the growth cone[11,30,31,32]. The semaphorins are a repulsive extracellular cue that collapses growth cones to repel them from inhospitable environments[33,34]. The semaphorin collapsin was found to function through both a Rho mediated signaling pathway and an endocytic pathway to enhance growth cone collapse[12]. Determination of the regulators of axonal guidance sheds insight into how the nervous system develops and obtains the highly ordered and complex network seen in the human body.

Acknowledgements

I would like to thank Dr. Shubhik DebBurman for his patience and unending support throughout the writing of this review. I would also like to thank Julie Wang for her editorial comments and Ray Choi for his friendship and supportive comments.

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